Development of Cell-based High Throughput Luminescence Assay for Drug Discovery in Inhibiting OCT4 and MAPKAPK2 Interaction

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Abstract

Overexpression of c-MYC protein without genomic amplification occurs in multiple cancers, including neuroblastoma and small cell lung cancer. In searching for the mechanisms of c-MYC protein overexpression, we demonstrated that the transcription factor, OCT4 mediates c-MYC transcriptional activation in progressive disease neuroblastoma. Subsequently, we identified two kinases, MAPKAPK2 (MK2) and DNA-PK, which are predicted to bind and phosphorylate OCT4 at S111 and S93 residues, respectively. Based on these novel observations, we developed a cell-based luminescence assay to screen and identify compounds that inhibit the interactions between MK2 and OCT4. By screening 79,671 compounds, we identified 65 compounds we designated as "hits". Using a two-step validation of co-immunoprecipitation and pOCT4S^{111} detection, the compounds were further narrowed down to three for further studies. The three compounds were tested for their ability to inhibit kinase activity, in vitro cytotoxic activity, and anti-inflammatory activity. In conclusion, we developed a cell-based luminescence assay for the discovery of new agents targeting the c-MYC transcriptional activation pathway. Screening and subsequent validation identified a small number of compounds for further development.

Keywords

Drug discovery, c-MYC, MAPKAPK2, protein-protein interaction, small molecule inhibitor, kinase

Introduction

Protein kinases phosphorylate serine, threonine, and tyrosine residues of other proteins to regulate cell signaling pathways of oncogenic transformation and metastasis, and the dysregulation of the pathways often results in cancer (Capra et al., 2006; Fiore, Forli, & Manetti, 2016; Kumar et al., 2010). Targeting kinase-associated pathways using small molecules became one of the essential strategies for cancer treatment (Gross, Rahal, Stransky, Lengauer, & Hoeflich, 2015; J. Zhang, Yang, & Gray, 2009). As of June 2020, 61 small molecule kinase inhibitors are approved by the FDA, and of those, only seven have non-cancer indications. To date, the majority of the FDA-approved drugs that inhibit protein kinases bind at the active site and compete with ATP. Depending on targeting mechanisms, the kinase inhibitors are classified into four types: ATP competitors, binders of the catalytic sites of the inactive conformation of kinases, binders of non-catalytic subunit/ATP-binding sites (also called allosteric inhibitors), reversible inhibitors of substrate binding sites, and covalent kinase inhibitors (Breen & Soellner, 2015). Despite their clinical success and FDA approval, such ATP-competitors have off-target effects due to low selectivity. This lack of selectivity can contribute to issues such as cardiotoxicity (Smyth & Collins, 2009). In addition, other types of kinase inhibitors reported

a higher degree of selectivity (Fiore et al., 2016). Efforts to develop kinase inhibitors that are independent of ATP-binding sites are led by industrial and academic laboratories (Kaoud et al., 2019; Kaoud et al., 2012).

c-MYC overexpression plays a vital role in the oncogenic transformation of cells in various cancers (Eischen, Roussel, Korsmeyer, & Cleveland, 2001; Kaur & Cole, 2013; Stine, Walton, Altman, Hsieh, & Dang, 2015). It is a transcription factor with a short half-life, and its expression is low and tightly expressed in normal cells (Dang, 2013; Farrell & Sears, 2014). On the other hand, either gene amplification or transcriptional/post-transcriptional regulation keeps the expression of c-MYC unusually high in tumor cells (Miller, Thomas, Islam, Muench, & Sedoris, 2012). Over a thousand c-MYC responsive genes have been reported, including genes involved in cell proliferation and metabolism (Miller et al., 2012). Given that c-MYC overexpression is frequent in cancers, and that it contributes to approximately 40% of all cancers in humans, regulation of c-MYC would benefit cancer treatment. However, strategies to directly target c-MYC have been challenging, mainly due to c-MYC being a transcription factor (Dang, Reddy, Shokat, & Soucek, 2017). Thus, different ways of targeting c-MYC should be developed.

Our group recently reported a novel c-MYC transcriptional activation pathway induced by OCT4 in the progressive disease of neuroblastoma (Wei et al., 2020). In the studies, we demonstrated that MAPKAPK2 (MK2) is one of two primary enzymes that phosphorylate OCT4 at serine 111 residue to induce c-MYC activation and that inhibiting the phosphorylation of OCT4 reduced c-MYC expression. To selectively inhibit the interaction of MK2 with OCT4, we identified fragments of OCT4 that are necessary to interact with MK2, and then used this knowledge to design a cell-based luminescence assay by exogenously expressing fragments of protein with luminescence probes. Using this assay, we screened a chemical library of compounds to identify "hits" that impede the MK2-OCT4 protein interaction in cells. Subsequently, we conducted hit validation studies. Here we describe this novel cell-based assay and the method of hit validation employed to identify a new category of OCT4/c-MYC modulators.

1. **Methods**
2. **Mammalian cell culture and transduction.**

HEK-293FT (ThermoFisher) cells were cultured in DMEM (ThermoFisher) supplemented with 10% FBS, 2 mM Glutamine, 100 units/mL Penicillin, 100 µg/mL Streptomycin Sulfate and 1 mM Sodium Pyruvate (ThermoFisher). The HEK-293FT cells were plated at a cell dose of 1×10^7on a 10-cm tissue culture dish and incubated at 37°C 5% CO_2 incubator until the cells reach 80% confluence. The cells were co-transfected either lentiviral ORFs, the constructs shown in Fig. 1 along with Lenti-vpak Packaging Kit (OriGene) using the transfection reagent MegaTran 1.0 (OriGene). After 48-72 hrs, the virus-containing medium was collected, spun down, filtered (0.45 µm), and used to infect target cells, NCI-H82. The virus-infected stable clones were obtained after selecting in 10% FBS/RPMI-1640 with 1 µg/mL of Puromycin (Sigma-Aldrich). The protein expression was confirmed in protein lysates extracted from the stable clones.

For cloning, *pLenti-C-myc-DDK-IRES-Puro* ,*pCMV6-POU5F1-mycDDK,* and *pCMV6-AN-HA-MAPKAPK2* were purchased from OriGene. To create the LgBiT-tagged truncated OCT4, the PCR amplified truncated *POU5F1* DNA fragment (nt1-648) was digested with *Sgf1* and *Mlu1-HF* and cloned into the P2A-tagged *pLenti-P2A-IRES-Puro* lentiviral vector to create the$pLenti\text{-}POU5F1^{nt1\text{-}648}\text{-}P2A$. Next, the pUCIDT-Kan vector (IDT) with synthesized LgBiT and 15GS (Gly-Ser) DNA linker sequences were inserted into the *Mlu1 -Sph1* sites of$pLenti\text{-}OCT4^{nt1\text{-}648}\text{-}P2A$($pLenti\text{-}OCT4^{nt1\text{-}648}\text{-}LgBiT\text{-}P2A$) by digesting with *Mlu1-HF* and *Sph1-HF* using the LigaFast™ Rapid DNA Ligation System (Promega). Furthermore, the *pCMV6-AN-HA-MK2* was cut with *EcoR1-HF* and*Sgf1* and then fused with *EcoR1-HA-Age1-Sgf1* linker double-stranded DNA sequence to create a unique *Age1* enzyme site. The pIDTSMART vector (IDT) with synthesized SmBiT and 15GS DNA linker sequences was cut with *Xho1* and *Pme1* and inserted into the COOH-terminus of *pCMV6-AN-HA-MK2* . Following digestion with*Age1-HF* and *Pme1,* the *MAPKAPK2-SmBiT* gene was ligated separately into the$pLenti\text{-}POU5F1^{nt1\text{-}648}\text{-}LgBiT\text{-}P2A$ to develop the$pLenti\text{-}POU5F1^{nt1\text{-}648}\text{-}LgBiT\text{-}P2A\text{-}MK2\text{-}SmBiT$construct. The DNA sequences of constructs were verified by Macrogen USA. The methods were performed in accordance with relevant guidelines and regulations and approved by the Institutional Biosafety Committee at Texas Tech University Health

Sciences Center. The current study did not involve animals or human subjects.

Chemical compounds

Approximately 80,000 compounds were provided by the Targeted Therapeutic Drug Discovery & Development Program at The University of Texas at Austin (Cho et al., 2018). The compounds were compiled from the following compound libraries: NIH clinical collection (674 compounds; Evotec, San Francisco, CA), Natural product or Natural product-like (3,280 compounds; MicroSource Discovery, Gaylordsville, CT and LifeChem, Niagara-on-the-Lake ON, Canada), Lopac (1,280 compounds; Sigma-Aldrich), fragment sets (18,143 compounds) obtained from Chembridge and ChemDiv, kinase set (11,250 compounds; Chembridge), and diversity sets (43,158 compounds) obtained from NCI, ChemDiv, LifeChem, and Maybridge (ThermoFisher). Additionally, two other libraries were interrogated: 1. A kinase-focused library (600 compounds), custom selected by the Texas Screening Alliance for Cancer Therapeutics (TxSACT) from various vendors with known activity against approximately 100 kinases, and 2. An academic collection (2,000 unique molecules) with diverse pharmacophores deposited from chemists at The University of Texas at Austin and the University of Kansas. Compounds were plated in 384-well plates dissolved in 100% DMSO at 10 mM concentration.

Identification of compounds interfering kinase-substrate binding ("Hit ID")

NCI-H82 cells stably expressing SmBiT-tagged MK2 and LgBiT-tagged OCT4 were suspended at 1 x 10^6 cells/mL of Opti-MEM® cell culture medium with reduced serum and seeded at 9 μL of the suspension per well in a sterile black 384-well plate (Greiner, Cat #788086). Then, the cells were incubated for ˜4 hours. Then 1 μL of the compounds (10 mM stock, 1:1,000 dilution with Opti-MEM®) was added to each well to give a final concentration of 1 μM. After 6 hrs of incubation with the compounds, Nano-Glo® Live Cell Assay (Promega) reagent was prepared as instructed by the company, and 1.3 μL added to each well. Then, the plates were incubated for 20-30 minutes at room temperature before luminescence was measured using SpectraMax iD3 microplate reader (Molecular Devices). The cell counts per well, incubation time, and serum content in the culture medium were optimized before the screening. All pipetting utilized a BenchSmart 96 semi-automated pipetting system (Rainin).

The luminescence was measured from each plate, and the data were collected as numerical values. For statistical analyses to identify a significant reduction in signals by compounds, data normality was tested by using a Shapiro–Wilk test and also visually examined by using a Q-Q normal plot. A box-cox transformation was performed when necessary. Compounds with a value of two standard deviations below the mean are considered outliers ("Hits"), i.e., inhibition of kinase-substrate binding, inhibition of kinase activity, or direct cell kill effect. The initial screening identifies compounds with any of these three effects.

Custom polyclonal phospho-OCT4^{S111} antibody production.

The anti-human phospho-OCT4^{S111}(anti-pOCT4^{S111}) rabbit antibody (RRID_AB_2721810) was produced by GenScript Biotech. The pOCT4^{S111}polyclonal antibody was prepared by immunizing two New Zealand rabbits three times with an NH_2-terminal KLH (keyhole limpet hemacyanin)-conjugated phosphopeptide SNSDGAPEPCTVT as an antigen. The phosphor-specific antibody was affinity-purified through a phosphopeptide-conjugated Sepharose CL-4B column. Eluted IgG was then passed through the corresponding non-phosphorylated peptide (SNSDGASPEPCTVT) column to deplete any IgG that was not specific to pOCT4^{S111}.

Validation of "Hits" by Immunoblotting and immunoprecipitation.

The stable cell line was prepared for immunoblotting and co-immunoprecipitation by infecting NCI-H82, a small cell lung cancer cell line, with a doxycycline-inducible *pCW57.1-POU5F1-mycDDK* construct using a lentiviral system as previously described (Wei et al., 2020; Y. Zhang et al., 2014) to validate the effect on kinase-substrate binding. Unless otherwise specified, cells grown in a T75 flask were first dissociated by PUCKs and washed once with ice-cold 1× PBS. Cells were lysed on ice with modified RIPA buffer, followed

by centrifugation at 14,000 × g for 15 min at 4°C. Protein concentration was determined by BCA assay (Pierce). An equal amount of proteins from different samples were electrophoretically separated on 4-12% SDS-PAGE, transferred to Hybond membrane (GE Healthcare), blocked with 1% BSA or 5% skim milk, immunoblotted with the indicated primary antibodies, and incubated with 1:3,000 HRP-conjugated mouse or rabbit IgG secondary antibodies followed by detection with enhanced chemiluminescence (GE Healthcare). The membrane was stripped and re-probed with an anti-GAPDH antibody to confirm equal protein loading. For immunoprecipitation, 500 μg of protein lysates as prepared above were pulled down at 4°C overnight with 40 μL EZview Red anti-FLAG M2 affinity gels (Sigma-Aldrich), washed 4 times with modified RIPA, and then eluted with an excess of 3× FLAG peptide (100 μg/mL) (Sigma-Aldrich) or incubated with 4x NuPAGE LDS sample loading buffer and 100 mM DTT. The immuno-complexes were denatured by heating at 95°C for 10 min and resolved by NuPAGE 4-12% SDS-PAGE and immunoblotted with the indicated antibodies.

In vitro **MK2 protein kinase activity assay**

For MK2 protein kinase assays, fully active, purified recombinant human GST-MK2 kinase enzyme (46-381 amino acids) (200 ng) (SignalChem) was incubated with the *E. coli* -expressed recombinant human full-length protein substrate His6-OCT4 (500 ng) (ProteinOne) or HSP27 (1 μg) (Enzo Life Sciences) at 30°C for 30 min in a pre-cooled microfuge tube with a total volume of 25 μL in 1x kinase reaction mixture containing 25 mM MOPS (pH=7.2), 12.5 mM β-glycerolphosphate, 25 mM $MgCl_2$, 5 mM EGTA, 2 mM EDTA, 0.25 mM DTT, and 100 μM ATP. For the test of the inhibition of the MK2 kinase activity, a specific MK2 inhibitor PF3644022 (1 μM) and the potential candidate compounds, including B5, C5, and E5 (1 μM each), were pretreated at 30°C for 10 min, respectively. The sterile-filtered DMSO was used as vehicle control. The reactions were quenched by the addition of 4x NuPAGE LDS sample loading buffer and 100 mM reducing agent DTT, followed by heating the samples at 70°C for 10 min. The protein samples were resolved on a NuPAGE 4-12% Bis-Tris SDS-PAGE gradient gel and then probed with the indicated antibodies.

Also, the MK2 protein kinase assays were performed using ADP-Glo^{TM} Kinase Assay Kits, as described in the instruction manual of Promega (#TM313) (Stokoe, Caudwell, Cohen, & Cohen, 1993). In brief, the kinase reaction was conducted at room temperature for 60 min in a total volume of 25 μL of 1x kinase buffer with components of fully active GST-MK2 enzyme (0.4 ng), HSP27tide synthetic peptide substrate RRLNRQLSVA-amide (0.4 μg), ATP (50 μM), and 1% DMSO vehicle or inhibitor. These inhibitors, including PF3644022 (100 nM or 1 μM), B5 (0.3 or 1 μM), C5 (30 nM or 100 nM), and E5 (10 nM or 30 nM), were pretreated prior to the addition of HSP27 synthetic peptide in the reaction mixture. After completion of the kinase reaction, the 25 μL of ADP-Glo Reagent was added and incubated at room temperature for 40 min to deplete the remaining ATP completely. After addition of the 50 μL of Kinase Detection Reagent for 30 min at room temperature, the ADP was converted into ATP, and the newly synthesized ATP was recorded and quantitated in luciferase/luciferin reaction using ELISA reader iD3 (Molecular Device). Data are shown as relative light units (RLU) that directly correlate to the amount of ADP produced.

In vitro **cytotoxic activity**

Human small cell lung cancer cell lines (NCI-H417, NCI-H82, NCI-H2171, NCI-H847, NCI-H1048, NCI-H146, NCI-H510A, NCI-H1963, NCI-H1876) were kindly provided by Dr. Adi Gazdar at University of Texas Southwestern. Cells were cultured in RPMI (GE Lifesciences) supplemented with 10% heat-inactivated fetal bovine serum. Cell lines used for the study were tested and free of mycoplasma. Cell line identities were verified using short tandem repeat genotyping as compared with the original primary sample material within the CCcells database: www.CCcells.org. Cells were plated in 96-well plates for 24 hours before treating with DMSO (0.1%), B5, C5, and E5 (1 nM – 10 μM in 3x increment). There were six replicates per drug concentration, and the control was treated with drug vehicle. After 96 hours of incubation with the compounds, cell viability was measured using the DIMSCAN assay, as described previously (Kang et al., 2011; C. Zhang et al., 2012).

Anti-Inflammatory Activity of the Compounds

THP-1, the human monocytic leukemia cell line, was cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Hsu et al., 2011). The cells were plated in 6-well plates at 3 x 10^6 cells/well in 2 mL culture medium and 100 ng/mL of PMA (Promega) for 48 h to induce differentiation. Then, PMA was washed twice with culture medium followed by another 40-48 hours of incubation. The culture medium was removed and replaced with 600 µL of culture medium containing the testing articles or vehicle control. After 2h, cells were treated with 100 ng/mL of*E. coli* -derived LPS (Sigma) for six additional hours, and supernatants were collected and stored at -80 °C until analysis. Immediately following the collection of the medium, cells were washed with ice-cold 1x PBS, lysed using 100 µL of lysis buffer before scraping the cells. The supernatants were spun for 10 minutes at 14,000 x g in a refrigerated centrifuge before transferring to microfuge tubes and storing in -80°C. IL-6 and TNF-α from the culture medium of cells were measured and quantified using BD OptEIA Human IL-6 ELISA Set and Human TNF-α ELISA Set kits (BD Biosciences) according to the manufacturer's instructions.

1. **Results**
2. **Assay development and optimization**

A schematic overview of the assay development and screening/validation assays is shown in Figure 1. NCI-H82 cells were stably transduced with a lentiviral vector construct to express both OCT4 and MK2 tagged with the luminescence probes, LgBiT and SmBiT, respectively (Figire 1). After selecting a subclone with puromycin, the screening assay was optimized for cell seeding (2,500, 5,000, and 10,000 cells/10 µL/well), incubation time in the presence of the luminescence substrate (continuously up to 45 min), and the content of fetal bovine serum (10% FBS in RPMI or Opti-MEM®). The luminescence signals were reduced in NCI-H82 cells cultured with RPMI with 10% FBS relative to Opti-MEM®. And 10,000 cells/well showed the highest luminescence (Figure S1), although the luminescence was reduced by 34% over 45 minutes.

Using the optimized luminescence assay, the compound library was screened for inhibition of the MK2-OCT4 interaction. This was achieved by measuring the reduction of the luminescence signal after a 20-30 minute incubation with the substrate (Figure 1). Of the 79,671 compounds tested, we identified 65 compounds that significantly reduced the luminescence (Figure 2). Since the purpose of the current study is the assay development to detect protein-protein interaction to screen compounds, the names and the chemical structures of the compounds identified as "hits" are not listed in this paper.

Effect on phosphorylation of OCT4 at S111

We previously showed that MK2 phosphorylates Ser111 residue of OCT4 (Wei et al., 2020). Thus, the inhibition of the phospho-$OCT4^{S111}$ levels was used as the first step of validation. To validate the effect of our compounds on the phosphorylation of $OCT4^{S111}$, we infected NCI-H82 cell line with a doxycycline-inducible lentiviral vector expressing*POU5F1* (the gene encoding OCT4) tagged with mycDDK. Using a custom-produced $pOCT4^{S111}$ antibody, we determined the effect of the "hits" on the phosphorylation of OCT4 at S111 residue in NCI-H82 cells with exogenous OCT4 overexpression (Figure S2A). After DOX-induction, followed by overnight treatment, immunoblot analysis was performed to determine the expression levels of $pOCT4^{S111}$. We identified three candidate compounds from the "hits" that significantly impaired phosphorylation of OCT4 at its Ser111 (Figure S2B & Figure 3A). In contrast, phosphorylation of HSP27, an MK2 substrate, was not affected by the compounds (Figure 3A). These compounds also decreased c-MYC expression, further confirming that the interaction between OCT4 and MK2 is targetable (Figure 3A).

Inhibition of MK2-OCT4 protein interaction

After validating the effects of the "hits" on the phosphorylation of OCT4 at Ser111 residue and c-MYC expression, we investigated the effect of the compounds targeting the protein-protein interaction between MK2 and OCT4. To this end, we used NCI-H82 stably infected with DOX-inducible *POU5F1* tagged with mycDDK (FLAG) constructs and treated these cells with our validated hits. We then pulled down the FLAG-tagged OCT4 protein and detected the presence of endogenous MK2. All three validated hits demonstrated a decreased association of MK2 after OCT4 pull-down at low nanomolar concentrations (Figure 3B). While previous assays showed that the phosphorylation of OCT4 at the Ser111 residue as well as c-MYC expression

was decreased, the stability of the OCT4 protein remained unchanged after pull-down, suggesting that the phosphorylation of OCT4 is related to the stability of the protein.

Characterization of compounds inhibiting MK2 enzymatic activity

To further explore the inhibitory mechanism of the compounds that impaired the ability of MK2 to phosphorylate OCT4, we used purified recombinant human GST-MK2 kinase and either *E. coli* -expressed recombinant human full-length His-OCT4 or HSP27 protein, a substrate of MK2. A known MK2 inhibitor, PF3644022, was used as a positive control. Compared with vehicle control, all three compounds reduced phospho-His-OCT4^{S111} levels. However, the three compounds did not affect the expression of pHSP27^{S78}, suggesting that none of the compounds affect the enzymatic activity of MK2 (Figure 3C). In the next experiment, the aim was to determine the inhibitory effect of the three compounds using an ADP-Glo^{TM} Kinase Assay. When GST-MK2 was incubated with HSP27tide, the MK2 kinase activity was inhibited by the positive control. In contrast, none of the three compounds significantly affect the ability of MK2 to phosphorylate HSP27tide (Figure 3D). These data indicate that the three compounds identified here are not typical inhibitors of MK2 enzymatic activity in preventing the phosphorylation of OCT4.

In vitro **cytotoxic activity**

The cytotoxicity of the three compounds was assessed in ten small cell lung cancer cell lines with various levels of c-MYC expression. IC_{50} concentrations of B5 ranged from 3 - 740 nM in the cell lines tested. The IC_{50} values of the C5 compound were 1–128 nM, and those of E5 was 1 – 9.9 nM (Figure 4A). The dose-response curves of E5 in all 9 cell lines are shown in Fig. 4b. IC_{50} values were lower than the lowest concentration tested (1 nM) in four of the 9 cell lines (Figure 4B). In three cell lines (NCI-H510A, NCI-H1963, NCI-H1876), the viability of cells treated with E5 was less than 10% (dose-response curves not included). All three compounds tested showed significant *in vitro* cytotoxicity at sub-nanomolar concentrations, depending on cell lines and the compounds.

Effect on Inflammatory Cytokines

As MK2 is a downstream substrate of the p38MAPK pathway and post-transcriptionally regulates cytokines, it is a pro-inflammatory mediator (Lee et al., 1994). Therefore, we evaluated the anti-inflammatory activity of the compounds at the concentrations with cytotoxic activity. While dexamethasone, a positive control, showed a significant reduction in TNF-α and IL-6 levels in a human monocytic leukemia cell line, no reduction in inflammatory cytokines was seen with the three compounds we identified, except under one condition: IL-6 level by 100 nM B5 (Figure 5A & B). These data suggest that the reduction of pOCT4^{S111} or c-MYC is not the consequece of the anti-inflammatory effect.

4. Discussion

In our previous study, we identified the MK2/OCT4/c-MYC axis as an*MYC* transcriptional activation pathway in progressive disease neuroblastoma (Wei et al., 2020). This novel mechanism for the regulation of c-MYC expression involves a kinase, and thus warrants studies to identify compounds targeting the pathway to regulate c-MYC. MK2 is activated by the p38 MAPK (p38) pathway, which has been investigated as a therapeutic target in inflammatory diseases due to its role in the regulation of TNF-α and other mediators with unknown immune responses (Geng, Valbracht, & Lotz, 1996). Despite the robust preclinical data, p38 inhibitors did not advance to phase III clinical studies due to the incidences of systemic toxicities, including hepato-, cardiac toxicities, and CNS disorders (Emami et al., 2015; O'Donoghue, Glaser, & Cavender, 2016), possibly due to the involvement of p38 in the regulation of more than 60 substrates with various physiological roles (Trempolec, Dave-Coll, & Nebreda, 2013). For this reason, MK2 (the first identified substrate of p38 (Fiore et al., 2016)) is being tested as an alternative target to p38 for treating inflammatory diseases (Fiore et al., 2016). Although targeting MK2 can potentially resolve some of these issues, this tactic may still face the issues that other kinase-targeting drugs experience, e.g. acquire resistance due to mutational changes in the kinase (Camidge, Pao, & Sequist, 2014; Holohan, Van Schaeybroeck, Longley, & Johnston, 2013; Rosenzweig, 2018). Also, many ATP-competitive kinase inhibitors affect multiple kinases, as they lack

specificity (Fiore et al., 2016; Gross et al., 2015). As described, we developed an assay to specifically target the c-MYC transcriptional activation pathway by targeting the interaction between MK2 and OCT4. Instead of developing the inhibitors of MK2 kinase, by targeting the interaction between the two proteins, the compounds inhibiting protein-protein interactions may be less susceptible to resistance than conventional kinase targeting drugs if the interfaces are critical for protein functions.

In identifying hits and validating them, several factors facilitated the steep selection process. First, to bypass an additional step of confirming cell penetration, a cell-based assay was implemented by exogenously expressing proteins in cancer cells. Thus, any compounds that are impermeable to cancer cells are eliminated at the first screening stage. Another strategy to expedite the process was to employ a lower initial concentration to limit the number of hits. Generally, the hit rate of a screening assay is approximately 0.4-1% (Newbatt et al., 2006; Yarrow, Totsukawa, Charras, & Mitchison, 2005). The hit rate of our assay was <0.1%, allowing for an efficient validation process.

Another unique feature of the screening method is the utilization of a single CMV promoter to generate the two proteins tagged with luminescence probes at an equal proportion. A commercially available product for a protein-protein interaction assay uses a bidirectional vector with two promoters to generate two proteins. This approach may produce two proteins with a different transcription/translation yield depending on the proficiency of the promoter. Of the two, the protein with a low molar concentration will be rate-limiting. To maximize the interaction and thus, the signals generated from the interaction, we used P2A, a peptide sequence of poliovirus, that cleaved the two proteins post-transcriptionally (Liu et al., 2017). Our experiments demonstrated that this strategy could be utilized to confirm the protein-protein interaction, which can be utilized in screening assays.

In conclusion, we developed an assay to screen and identify compounds that inhibits the interaction of MK2 and OCT4, which is a new c-MYC transcriptional activation mechanism that we previously identified. A two-step validation narrowed the initial hits to three compounds, and all three compounds appear to show *in vitro* anti-cancer activity at nanomolar concentrations. The current process demonstrated that the initial drug development procedure could contract several steps into one or two, and it may be suitable for the early stages of drug development in laboratories without costly equipment or reagents.

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Conflict of Interest:

The authors have no conflict of interest to declose.

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Figure Legends

Figure 1. Schematic of initial screening and two-step hit validation. Initial screening was done after the transduction with wild-type *POU5F1* (nt1-648) tagged with LgBiT and *MAPKAPK2*(full length) tagged with SmBiT in NCI-H82 cells. Optimal orientation of LgBiT and SmBiT was determined by stably transducing with various constructs endocing fluorescence probes and proteins in different sequences and comparing the luminescence. Once stable clones were established, cells were seeded in a 384-well plate

and were treated with an inhibitor. Decreased luminescence indicated a positive hit. Screening and hit identification yielded 65 compounds that were selected for further study. These compounds were validated via determining their effect on phosphorylation of $OCT4^{S111}$, kinase activity, and the protein-protein interaction between OCT4 and MK2. Three compounds were deemed to inhibit $pOCT4^{S111}$, and c-MYC expression, impair MK2 kinase activity, and prevent the protein-protein interaction between OCT4 and MK2. Docking studies and*in vitro* activity testing was conducted on these 3 compounds.

Figure 2. Compound screening and identification of "hits." **a**Graphical representation of plates used to identify hits as potential inhibitors of MK2-mediated phosphorylation of OCT4. A blank plate (top) was used to standardize readings. Compounds that demonstrated significant decreases in luminescence are shown in red (middle and bottom). **b** Compounds were deemed as hits (red triangles) if they demonstrated a reduction in luminescence greater than 1.96 x the standard deviation from the mean as calculated in the blank plate panel (top). Red triangles represent significant inhibition (1.96 x the standard deviation).

Figure 3. Hit validation, *in vitro* assays, and kinase activity assays. **a** Hit validation demonstrating that three compounds: B5, C5, and E5 inhibit OCT4, $pOCT4^{S111}$, and c-MYC expression, but not phosphorylation of HSP27, in a DOX-inducible OCT4-overexpressing SCLC cell line (NCI-H82 pCW57.1-*POU5F1* -mycDDK). Followed by DOX-induction, the cells for 8 hrs with the compounds at 2 µM, and subjected to immunoblotting.**b** Hit validation by pull-down studies in the DOX-inducible OCT4-overexpressing SCLC cell line demonstrates that treatment with B5, C5, and E5 disrupt the protein-protein interaction between OCT4 and MK2 in a dose-dependent manner. **c** *In vitro* kinase activity assays. MK2 (GST-tag) was incubated with inhibitors for 30 minutes before the addition of bacterially-derived OCT4 (His-tag) or HSP27 protein. After a second 30 minute incubation, IB demonstrates that $OCT4^{S111}$ phosphorylation, but not $HSP27^{S78}$ phosphorylation, was decreased. PF3644022 (known ATP-competitive MK2 inhibitor) was used as a positive control.**d** ADP-Glo^{TM} assay demonstrates that B5, C5, and D5 do not affect MK2 kinase activity.

Figure 4. *In vitro* cytotoxicity assays. **a** Calculated IC_{50} values for B5 (left), C5 (middle), and E5 (right) in 9 high and low c-MYC-expressing SCLC cell lines. **b** E5 demonstrates cytotoxic activity towards 9 SCLC cell lines. Similarly, B5 and C5 demonstrate significant activity (not shown). In three cell lines (NCI-H510A, NCI-H1963, NCI-H1876), the viability of cells treated with E5 were less tan 10%, and thus the dose-response curves are not shown. The cells were treated with vehicle (DMSO, 0.1% as final concentration), 1 nM, 3 nM, 10 nM, 30 nM, 100 nM, 300 nM, 1 µM, 3 µM, 10 µM for 96 hrs before viability was assessed. Each condition was tested in 6 replicates. Symbols: mean, error bars: standard deviation.

Figure 5. ELISA assay confirming that validated compounds do not inhibit the production of inflammatory cytokines. **a** TNF-α production was unchanged after inhibitor treatment. Dexamethasone was used as a positive control. **b** Similarly, IL-6 production was unaffected after treatment with inhibitors.

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